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16. ABSTRACT

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Manganese Chloride Enhances Natural Cell-Mediated Immune Effector Cell Function: Effects on Macrophages

Ralph J. Smialowicz, Robert W. Luebke, Ronald R. Rogers, Marie M. Riddle and Denise G. Rowe

Immunobiology Section, Perinatal Toxicology Branch, Developmental Biology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, U.S.A.

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Abstract: A single intramuscular injection of $MnCl_2$ in mice caused an increase in macrophage functional activity. Spleen cell antibody-dependent cell-mediated cytotoxicity against both chicken erythrocytes and P815 tumor cell targets was enhanced 24 h following a single injection of $MnCl_2$. Enhanced antibody-dependent cell-mediated cytotoxicity activity following $MnCl_2$ treatment was not associated with a change in spleen cellularities compared with saline-injected mice. Resident peritoneal macrophages from mice injected intramuscularly with $MnCl_2$ displayed enhanced phagocytic activity for chicken erythrocytes in the presence or absence of opsonizing antibody. Enhanced cytolytic activity against P815 mastocytoma target cells and enhanced cytostatic activity against MBL-2 lymphoma target cells was also observed for nonelicited resident peritoneal macrophages from mice injected intramuscularly with $MnCl_2$. There were no differences in the cellularity or relative number of adherent cells obtained from the peritoneal cavity of saline or $MnCl_2$-injected mice. These enhanced macrophage functions were associated with the induction of increased interferon levels in mice injected with $MnCl_2$.

Key words: Manganese chloride; Macrophages; Phagocytosis; Cytotoxicity; Interferon

Introduction

A variety of metals have been shown to be potent immunomodulators (Spallholz et al., 1976; Koller, 1980; Lawrence, 1981; Beach et al., 1982; Neilan et al., 1983; Smialowicz et al., 1984a). Recently, we described the immuno-enhancing effect of $MnCl_2$ on natural killer (NK) cell activity in mice (Rogers et al., 1983; Smialowicz et al., 1984b). A single intramuscular injection of $MnCl_2$ caused a significant enhancement of spleen NK cell activity as measured in a 4-h in vitro ^{51}Cr release assay. Enhanced NK activity persisted for up to 4 days, was associated with a nonadherent spleen cell population, and was completely eliminated by injection with antiasialo GM1 serum (Smialowicz et al., 1984b). Manganese enhanced NK activity in several mouse strains with differing NK cell reactivity and with several tumor target cells differing in sensitivity to NK cytolysis. The enhancement of NK activity in mice injected with $MnCl_2$ was accompanied by an increase in circulating interferon (IFN) levels. Rabbit anti-mouse IFN α, β but not anti-mouse IFNγ completely eliminated the $MnCl_2$-enhanced NK cell activity in the spleens of mice (Smialowicz et al., 1984b). A single intramuscular injection of $MnCl_2$ also enhanced the clearance of radiolabeled tumor cells from the lungs and spleen of mice (Rogers et al., 1983) and inhibited the growth of B16-F10 melanoma lung tumors (Smialowicz et al., 1984b).

Abbreviations: (N)K, (natural) killer; ADCC, antibody-dependent cell-mediated cytotoxicity; PC, peritoneal cells; CRBC, chicken red blood cells; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LPS, lipopolysaccharide; IFN, interferon; HBSS, Hank's balanced salt solution.

It is well known that IFN has immunomodulating properties that affect other cells of the immune system in addition to NK cells (De Maeyer and De Maeyer-Guignard, 1982). IFN is known to enhance various macrophage functions including phagocytosis (Huang, 1978) and cytotoxic activity against tumors (Borachi et al., 1982). In the light of our earlier work, which demonstrated that $MnCl_2$ enhanced NK activity by the induction of interferon, we decided to examine the effects of $MnCl_2$ on macrophage function. In the work reported here we present evidence that $MnCl_2$ enhances spleen cell ADCC activity and resident peritoneal macrophage phagocytic, cytolytic and cytostatic activity.

Materials and Methods

Mice

Eight- to twelve-week-old C57BL/6J female or CBA/J male mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were used. Mice were maintained in our vivarium for at least 1 week after arrival to allow recovery from shipping stress.

Manganese chloride

A stock solution of $MnCl_2$ ($MnCl_2 \cdot 4H_2O$; Fisher Scientific, Fair Lawn, NJ) was prepared in sterile nonpyrogenic normal (0.9%) saline. The manganese concentration was determined by neutron activation analysis (Dr. J. N. Weaver, North Carolina State University, Raleigh, NC). For injection, aliquots of the $MnCl_2$ stock solution were diluted to the desired concentration in sterile saline buffered with 25 mM HEPES. Mice were given a single intramuscular injection in the thigh on a body weight basis 24 h prior to assay. The injection volumes ranged from 0.10 to 0.15 ml. Control mice were injected with equivalent volumes of HEPES-saline.

IFN assay

IFN (α, β and γ) levels in mouse serum were determined by inhibition of the cytopathic effect of vesicular stomatitis virus on murine L929 cells (Biofluids Inc., Rockville, MD).

ADCC assays

ADCC assays against chicken red blood cells (CRBC) and P815 mastocytoma target cells (DBA/2, American Type Culture Collection No. TIB-64, Rockville, MD) were performed using mouse spleen cells as described by Pollack (1977) and Kumagai et al. (1982), respectively. Briefly, CRBC and P815 cells were labeled with ^{51}Cr (50 μCi ^{51}Cr as sodium chromate/10^6 cells; New England Nuclear, Boston, MA) and resuspended to 4 $\times$ 10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Grand Island Biological Co.). Mouse anti-CRBC, anti-P815 or normal mouse sera were added to labeled CRBC or P815 cells as appropriate. The final antisera dilutions were 1:1000 anti-CRBC and 1:200 anti-P815. Antiserum-coated ^{51}Cr-CRBC or ^{51}Cr-P815 cells (4 $\times$ 10^5 in a volume of 100 μl) were added to graded numbers of splenic effector cells (100 μl) in round-bottom microtiter plates (Linbro). Three replicates of each effector to target cell ratio (12.5:1, 25:1 and 50:1) were incubated for 4 h at 37°C, 5% CO_2. Target cells treated with nonimmune mouse serum, which served as a control, were added to effector cells at a 50:1 ratio. Supernatants were collected and the percent specific ^{51}Cr release was determined according to the equation:

$$^{51}\text{Cr release} = \left(\frac{E - S}{T - S}\right) \times 100\%$$

where E is the ^{51}Cr released from target cells in the presence of spleen cells, S is spontaneous release of ^{51}Cr from target cells alone, and T is the maximum release of ^{51}Cr from target cells in the presence of 0.25% Triton X-100.

Phagocytosis

Non-elicited resident peritoneal cells (PC) were obtained by using sterile Hank's Balanced Salt Solu-

tion (HBSS; GIBCO) containing 10 units/ml sodium heparin as described (Smialowicz et al., 1984a). Phagocytosis of ^{51}Cr-CRBC by PC was determined with modification of the procedure described by Walker and Demus (1975). 5×10^5 PC in 0.5 ml RPMI 1640 with 10% heat-inactivated γ globulin-free fetal bovine serum (GIBCO) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), was added to 16-mm diameter wells of a 24-well culture plate (Costar, Cambridge, MA). Cultures were incubated at 37°C in 5% CO_2 for 2 h to allow macrophages to adhere and then washed three times with warm HBSS to remove nonadherent cells. Cultures were incubated for an additional 1 h in 0.5 ml of supplemented RPMI 1640 medium. Medium was removed and 5×10^5 ^{51}Cr-CRBC and diluted (1:1000) anti-CRBC serum or normal mouse serum was added to macrophage monolayers in a total volume of 0.5 ml/well. Phagocytosis was determined at 0, 30, 60 and 120 min. Culture plates were centrifuged for 5 min at 250 × *g*, after which the supernatant was collected and counted in a gamma spectrometer. Distilled water (0.5 ml) was then added to culture wells, mixed and allowed to stand for 5 min at room temperature to lyse CRBC adhering to macrophages. The culture plates were recentrifuged and the supernatant removed and counted. Finally, the cell monolayer was lysed by the addition of 0.5 ml of 0.1 N NaOH and the lysate counted. The macrophage phagocytic activity (*P*) in each culture was estimated by determining the percentage of total radioactivity associated with the cell monolayer following the water-lysis step:

$$P = \frac{^{51}\text{Cr released from NaOH lysate}}{\text{Total } ^{51}\text{Cr in culture}} \times 100\%.$$

Cytolysis

The cytolytic activity of resident PC against P815 mastocytoma cells was performed with modification of that described by Taffet and Russell (1980). PC suspended in supplemented RPMI 1640 were seeded in quadruplicate at 2, 4, 6 and 8×10^5 cells /well. The plates were incubated for 2 h at 37°C in 5% CO_2 and nonadherent cells were removed from the wells by washing 3 times with warm medium. Approximately 50% of PC adhered to culture wells. P815 cells (2×10^4) labeled with ^{51}Cr were then added to the wells resulting in initial effector to target cells ratios of 5, 10, 15 and 20:1. After 18 h of culture the plates were centrifuged for 5 min at 250 × *g* and the supernatant was removed using the Skatron Supernatant Collection System (SCS; Skatron Inc., Sterling, VA) and counted. Results were expressed as percent specific ^{51}Cr release as described above for the ADCC assay.

Cytostasis

The ability of resident PC to inhibit the growth of MBL-2 Moloney virus-induced lymphoma cells (obtained from Dr. M. Luster, National Institute of Environmental Health Sciences, R.T.P., NC) was determined with modification of the technique described by Dean et al. (1978). Quadruplicate flat-bottom wells of a microtiter plate (No. 3596, Costar) were seeded with 2 and 4×10^5 PC/well. The plates were incubated for 2 h at 37°C in 5% CO_2 and nonadherent cells were removed from the wells by washing 3 times with warm medium. Approximately 50% of PC adhered to the culture wells. To each well was added 0.1 ml of medium containing 2×10^4 MBL-2 cells from a 48 h culture. This corresponds to initial effector to target cell ratios of 10:1 and 20:1. To other wells (at least 12) containing no macrophages were added 2×10^4 MBL-2 cells which served as controls for determining maximum labeling. Cultures were incubated for 48 h at 37°C in 5% CO_2. Cultures were washed to remove excess PC-derived thymidine 6 h before harvesting and then pulsed with 0.5 μCi of [*methyl* ^{3}H]thymidine (specific activity 6.7 Ci/mM; New England Nuclear). Cells were harvested with a MASH II automated harvester (Microbiological Associates, Bethesda, MD). Percent cytostasis was determined by the following equation:

$$\% \text{ Cytostasis} = \left(1 - \frac{(A - C)}{B}\right) \times 100$$

where A is the cpm of cultures containing effector and target cells, C is the cpm of cultures containing only effector cells, and B is the cpm of cultures containing only target cells.

Quantitation of macrophages

Enumeration of adherent cells (macrophages) in the phagocytosis, cytotoxicity and cytostasis assays was done using a modification of the technique described by Nakagawara and Nathan (1983). Briefly, after removal of nonadherent cells from microtiter wells, 100 μl of a solution containing 1% Triton X-100 in 0.1 M citric acid with 0.05% (w/v) naphthol blue black (Allied Chemical Co., New York, NY) was added to each well. After 30 min at room temperature, the plates were placed on a vibrating platform for 5 min. The solution within each well was further mixed and transferred to a hemacytometer. The fixed and stained nuclei were then counted and the number of adherent cells/well was determined. Identification of macrophages within the adherent population was based upon morphological criteria of Giemsa-stained adherent PC on tissue culture chamber slides (Lab-Tek, Miles Laboratories, Naperville, IL) and phagocytosis of 1.01-μM diameter polystyrene latex particles (Smialowicz and Schwab, 1978).

Results

ADCC activity

A single intramuscular injection of as little as 10 μg/g $MnCl_2$ caused a significant ($P < 0.05$) enhancement of spleen cell ADCC activity against CRBC targets 24 h later (Table I). The $MnCl_2$ doses which enhanced ADCC in this experiment were comparable to those which enhanced splenic NK cell activity (Smialowicz et al., 1984b). Table II

TABLE I

$MnCl_2$ enhances spleen cell antibody-dependent cell-mediated cytotoxicity against chicken erythrocytes

Treatment[a]	Dose (μg/g)	% ^{51}Cr release[b] at E:T ratio of:		
		12.5:1	25:1	50:1
Saline		14.4 ± 1.3	19.2 ± 1.4	21.0 ± 1.2
$MnCl_2$	10	21.4 ± 1.9*	27.5 ± 1.3*	27.0 ± 1.3*
	20	23.9 ± 1.5*	28.2 ± 2.0*	28.2 ± 3.1*
	40	24.0 ± 1.4*	23.6 ± 2.4*	20.9 ± 1.1

[a] C57BL/6J mice were given a single intramuscular injection 24 h prior to assay (n = 10/group).
[b] ADCC activity was determined in the presence of mouse anti-CRBC serum. Values given are mean ± SE.
* Significantly different from saline control ($p < 0.05$; Dunnett's t-test).

TABLE II

Comparison of the effect of $MnCl_2$ on spleen cell antibody-dependent cell-mediated cytotoxicity against two different target cells

Target	Treatment[a]	% ^{51}Cr release[b]			
		Anti-serum			Normal serum
		12.5:1[c]	25:1	50:1	50:1
CRBC	Saline	15.6 ± 0.9	20.8 ± 0.8	20.3 ± 0.9	0.3 ± 0.1
	$MnCl_2$	25.7 ± 0.5*	24.5 ± 0.7*	18.7 ± 0.5	0.4 ± 0.1
P815	Saline	3.9 ± 0.6	5.5 ± 0.7	7.7 ± 0.7	1.8 ± 0.5
	$MnCl_2$	14.0 ± 0.9*	18.0 ± 1.3*	25.0 ± 1.3*	6.4 ± 0.9*

[a] Groups of 8 C57BL/6J mice were given a single intramuscular injection with saline or 40 μg/g $MnCl_2$ 24 h prior to assay.
[b] Antibody-dependent cell-mediated cytotoxicity was performed in the presence of mouse anti-serum to CRBC or P815 or with normal mouse serum. Values given are mean ± SE.
[c] Effector to target ratio.
* Significantly different from saline control ($p < 0.01$; Student's t-test).

shows the results of an experiment in which the ADCC activity of spleen cells from $MnCl_2$-injected mice was determined using CRBC or P815 mastocytoma cells as targets. A significant enhancement in ADCC activity of spleen cells from $MnCl_2$-injected mice was observed against both targets. There was no difference in the number of nucleated spleen cells between control and $MnCl_2$-treated mice (data not shown). The significant enhancement of cytotoxicity against P815 targets in the absence of antibody (Table II) may represent splenic natural killer cell activity.

Low levels of IFN were detected in the sera of mice injected 24 h previously with $MnCl_2$. IFN levels for saline-injected mice and mice injected intramuscularly with 20, 40 or 80 μg/g $MnCl_2$ were 0, 10, 30 and 55 IU/ml respectively ($n = 6$). These circulating levels of IFN correspond to those obtained in earlier experiments (Rogers et al., 1983; Smialowicz et al., 1984b).

Phagocytosis

$MnCl_2$ enhanced the phagocytic activity of resident peritoneal macrophages from CBA/J mice in the presence or absence of opsonizing antibody (Fig. 1). Significantly greater uptake of ^{51}Cr-CRBC by macrophages from $MnCl_2$-injected mice was observed in the presence of antibodies after 30 min of incubation and in normal serum after 60 min of incubation. Table III summarizes the results from three separate experiments in which enhanced phagocytosis was observed for macrophages from $MnCl_2$-injected C57BL/6J mice compared with macrophages from saline control mice. While enhanced phagocytosis was observed in all three of these experiments, significantly greater ($p < 0.05$) uptake of ^{51}Cr-CRBC was observed only in experiments 1 and 3.

Cytolysis and cytostasis

The effects of a single intramuscular injection of $MnCl_2$ on resident peritoneal macrophage cytolytic activity against P815 mastocytoma cells and cytostatic activity against MBL-2 mouse lymphoma cells are shown in Tables IV–VI. Table IV shows the results from several early experiments in which both

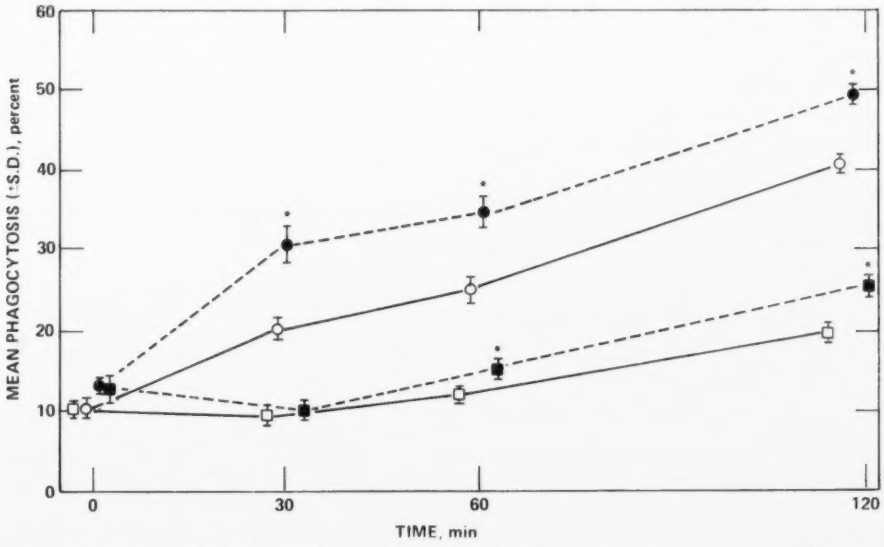

Fig. 1. $MnCl_2$-stimulated macrophage phagocytosis. Resident peritoneal macrophages were cultured for 24 h following a single intramuscular injection of saline or 80 μg/g $MnCl_2$ in CBA/J mice. Data are the mean of triplicate cultures for each time with saline plus mouse anti-CRBC serum (○), $MnCl_2$ plus mouse anti-CRBC serum (●), saline plus normal mouse serum (□) and $MnCl_2$ plus normal mouse serum (■); * indicates $p < 0.05$ according to Student's t-test.

TABLE III

The effect of $MnCl_2$ on macrophage phagocytic capacity

Experiment	Treatment[a]	n	% Phagocytosis[b]
1	Saline	4	40.9 ± 3.4
	$MnCl_2$	4	56.9 ± 4.4*
2	Saline	4	46.6 ± 3.0
	$MnCl_2$	4	53.7 ± 1.7
3	Saline	4	45.3 ± 3.1
	$MnCl_2$	3	57.5 ± 1.6*

[a] Groups of 10 C57BL/6J mice were given a single intramuscular injection 24 h prior to assay. PCs were pooled and quadruplicate wells were prepared. $MnCl_2$ was administered at 40 μg/g.

[b] Phagocytosis was determined using ^{51}Cr-CRBC in the presence of opsonizing antibody for a period of 60 min. Data are the mean of 4 replicate wells for each of 3 4 separate pools of PC. Values given are mean ± SE.

* Significantly different from saline control ($p < 0.05$; Student's t-test).

the cytolytic and cytostatic capacity of macrophages from Mn-injected mice were observed to be enhanced.

The effect that the number of macrophages in culture has on the cytotoxicity of peritoneal cells from Mn-injected mice is shown in Table V. Doe and Henson (1978) reported that lysis of target cells by macrophages is dependent upon the formation of a confluent monolayer. Confluent macrophage monolayers were observed microscopically when 6-8 × 10^5 PC were added to each tissue culture well (i.e. at effector to target ratios of 15:1 and 20:1 respectively). At these ratios as well as at an effector to target ratio of 10:1, significant enhancement was observed in the macrophage lytic activity of cells obtained from mice receiving 40 μg/g $MnCl_2$ (Table V). This result corresponds well with the results of Doe and Henson (1978), who reported enhanced macrophage lytic activity at effector to target ratios greater than 10:1.

It is well known that bacterial endotoxins are capable of stimulating macrophages to become nonspecifically cytolytic or "activated" for killing tumor cells (Alexander and Evans, 1971). Endotoxins are also potent IFN inducers (Ho and Armstrong, 1975). In an attempt to clarify whether $MnCl_2$ or endotoxin was responsible for the ob-

TABLE IV

The effect of $MnCl_2$ on macrophage cytolysis and cytostasis

Treatment[a]	% Cytolysis[b]	% Cytostasis[b]	
	10:1[c]	10:1[c]	20:1
Saline	−0.1 ± 0.5	20.4 ± 2.7	33.6 ± 4.2
$MnCl_2$	32.6 ± 16.2*	65.0 ± 7.1*	93.2 ± 2.7*

[a] Groups of 10 C57BL/6J mice were given a single intramuscular injection of saline or 40 μg/g $MnCl_2$ 24 h prior to assay. PCs were pooled and quadruplicate wells were prepared.

[b] Mean (± SE) of 2 separate experiments for cytolysis and 3 separate experiments for cytostasis.

[c] Effector: target cell ratio with P815 targets for cytolysis and MBL-2 targets for cytostasis.

* Significantly different from saline controls ($p < 0.01$; Student's t-test).

TABLE V

$MnCl_2$ enhances macrophage cytolytic capacity against P815 targets

Treatment[a]	% Cytolysis[b] at E:T ratio of:			
	5:1	10:1	15:1	20:1
Saline	−1.3 ± 0.9	1.4 ± 0.7	10.7 ± 1.3	11.5 ± 2.2
$MnCl_2$	−0.1 ± 1.4	15.1 ± 3.3*	30.9 ± 1.2*	28.9 ± 1.6*

[a] Groups of 10 C57BL/6J mice were given a single intramuscular injection of saline or 40 μg/g $MnCl_2$ 24 h prior to assay. PCs were pooled and quadruplicate wells were prepared for each of 3 separate groups.

[b] Values are mean ± SE.

* Significantly different from saline control ($p < 0.01$; Student's t-test).

TABLE VI

The effect of $MnCl_2$ on macrophage cytolytic and cytostatic capacity against tumor cells in endotoxin-free serum

Treatment[a]	Dose (μg/g)	% Cytolysis[b,c] at E:T ratio of:			% Cytostasis[b,c] at E:T ratio of:	
		10:1	15:1	20:1	10:1	20:1
Saline		0.7 ± 1.6	5.3 ± 2.0	11.7 ± 5.5	6.9 ± 11.8	56.1 ± 20.9
$MnCl_2$	20	0.2 ± 0.6	10.1 ± 0.4	33.5 ± 3.0*	28.7 ± 9.3	64.2 ± 14.5
	40	0.2 ± 1.3	13.2 ± 1.0	28.0 ± 5.9	38.1 ± 8.5	79.1 ± 16.1
	80	1.7 ± 2.1	23.9 ± 3.4*	37.6 ± 4.7*	31.8 ± 7.7	86.4 ± 5.5

[a] Groups of 10 C57BL/6 J mice were given an intramuscular injection 24 h prior to assay. PCs were pooled and quadruplicate wells were prepared for each of 3 separate groups.
[b] Cells were cultured in endotoxin-free fetal bovine serum (Hy Clone Labs, Sterile Systems Inc., Logan, UT).
[c] Values are mean ± SE.
* Significantly different from saline control ($p < 0.05$; Dunnett's t-test).

served enhancement of macrophage tumoricidal activity during in vitro cultivation, experiments were performed using "endotoxin-free" serum obtained from Hy Clone Labs (Sterile Systems, Inc., Logan, UT). The results of one such study are shown in Table VI. Once again $MnCl_2$ enhanced both the cytolytic and cytostatic activity of nonelicited resident peritoneal macrophages from Mn-injected mice. In addition, the $MnCl_2$ stock solution was found to be free of endotoxin as determined by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO).

We observed a great degree of variability in the macrophage cytolytic and cytostatic assays employed in these studies. As can be seen in Table VI, while enhancement of cytostasis was observed following Mn-treatment, it was not significantly greater than the controls at the $p < 0.05$ level. Nevertheless, in 16 separate experiments, the cytotoxic activity of resident peritoneal macrophages was always greater than or equal to that of controls.

Enhanced macrophage function following $MnCl_2$ injection was not due to greater numbers of cells obtained from the peritoneal cavity of these mice, nor was it due to a difference in the number of adherent cells after several hours in culture. The

TABLE VII

Characteristics of resident peritoneal macrophages following $MnCl_2$ treatment in vivo

Treatment[a]	Dose (μg/g)	Peritoneal cells[b] ($\times 10^6$)	Adherent cells[c] ($\times 10^5$)		% Latex phagocytosis[d]
			2 hours	18 hours	
Saline		26.5 ± 1.9	1.86 ± 0.07	1.18 ± 0.11	94.6 ± 2.0
$MnCl_2$	20	28.1 ± 4.5	1.97 ± 0.10	1.42 ± 0.06	97.5 ± 0.5
	40	22.9 ± 1.3	1.75 ± 0.20	1.41 ± 0.16	96.7 ± 0.5
	80	20.2 ± 0.9	1.55 ± 0.23	1.42 ± 0.25	96.7 ± 1.7

[a] Groups of 10 C57BL/6J mice were given an intramuscular injection 24 h prior to assay.
[b] Mean number of peritoneal cells obtained from 3 groups of 10 mice/group.
[c] PCs were pooled and quadruplicate wells were prepared at 4×10^5 cells/well; 2 h later culture wells were vigorously washed and the number of adherent cells was determined for 3 separate sets of quadruplicate wells for each treatment. The number of adherent cells in an equal number of culture wells were determined 18 h later.
[d] Mean percent adherent cells phagocytizing latex particles over a 60 min incubation ($N = 3$).
All valves given represent mean ± SE.

results from a representative experiment are shown in Table VII. No differences were observed between saline controls and the t ·ree $MnCl_2$ doses for the following: (1) the number of peritoneal cells obtained; (2) the number of adherent cells/well after 2 and 18 h in culture; and (3) the percentage of adherent cells that phagocytized latex particles. These results indicate that the observed enhanced macrophage function was due to an intrinsic "activation" of the macrophages by $MnCl_2$ and not due to an increase in their numbers.

Discussion

Macrophages can be activated to a tumoricidal state and to an increased phagocytic activity by a number of interferon inducers including lipopolysaccharide (LPS), certain viruses, synthetic double stranded RNA (Poly I:C), pyran copolymer, and certain bacteria (Schultz et al., 1977; Schultz, 1980). In the work presented here, $MnCl_2$ was found to enhance macrophage phagocytic activity as well as tumoristatic and tumoricidal activity. This enhanced macrophage activity was accompanied by the induction of low levels of circulating IFN. In our earlier work (Rogers et al., 1983; Smialowicz et al., 1984b) enhanced NK activity following $MnCl_2$ treatment was also associated with the induction of interferon.

While macrophages become tumoricidal upon exposure to IFN-inducing agents individually, optimal macrophage activation is believed to involve the action of two agents or signals administered in a particular sequence (Pidgeon et al., 1983). For example, LPS is required as a second signal for lymphokine (macrophage activating factor)-induced cytotoxicity. Such a second signal for macrophage activation has been reported not to be required for Poly I:C-induced cytotoxicity (Taramelli and Varesio, 1981; Taramelli et al., 1983) although a synergism between Poly I:C and macrophage activating factor has been reported by others (Pidgeon et al., 1983). The exact mechanism whereby $MnCl_2$ enhances macrophage functional activity is not completely clear, although a role for interferon is strongly suggested. Whether other factors, such as lymphokines or other soluble mediators, play a role in Mn-induced macrophage activation remains to be determined. Nonspecific inflammation, which occurs at the site of Mn injection, may also play a role in the expression of macrophage activation at a site (i.e. peritoneal cavity) removed from Mn treatment.

Although a role for LPS in concert with interferon cannot be completely ruled out as a possible mechanism in the present work, it seems highly unlikely. Evidence for this suggestion comes from experiments in which endotoxin-free serum was employed in the culture medium used in the cytolysis and cytostasis assays (Table VI). Further evidence refuting a requirement for LPS comes from our earlier work in which we determined that LPS was not responsible for NK enhancement because C3H/HeJ mice, which are refractory to LPS-induced NK augmentation, displayed enhanced NK activity following Mn treatment (Smialowicz et al., 1984b). Furthermore, the absence of endotoxin contamination in our NK work was verified using the *Limulus* amebocyte lysate assay (Smialowicz et al., 1984b).

Other evidence against a role for LPS in the observed enhancement of macrophage function by $MnCl_2$ is the fact that ADCC activity of spleen cells was enhanced following Mn treatment. Folks and Schlagel (1982) recently reported that LPS suppressed the expression of ADCC activity of spleen cells against CRBC following administration in vivo or when added to cultures in vitro. In the present work, ADCC activity against both CRBC and P815 mastocytoma cells was enhanced 24 h following a single intramuscular injection of $MnCl_2$.

It has been postulated that at least two different spleen cell types, which can be distinguished by the target cells employed, may serve as effector cells in ADCC. Cells of the macrophage monocyte lineage which are adherent, phagocytic, and have receptors for the Fc fragment of Ig (FcR +) mediate cytolysis of antibody-coated erythrocytes. On the other hand, nonadherent nonphagocytic FcR + lymphocytes or killer (K) cells are cytolytic toward antibody-coated tumor cells (Greenberg et al., 1975;

Pollack, 1977; Pollack and Kraft, 1977; Santoni et al., 1979). ADCC activity against both tumor cell and erythrocyte targets has been reported to be enhanced by interferon (Santoli et al., 1978; Attallah and Folks, 1979; Kaminsky et al., 1983). Since $MnCl_2$ induces IFN, and LPS inhibits ADCC activity against CRBC, these results suggest that $MnCl_2$ is not only enhancing macrophage-mediated ADCC, but also K cell-mediated ADCC through the induction of interferon.

It is interesting to note that enhanced cytotoxicity was observed for spleen cells from Mn-treated mice against P815 targets in the absence of antibody (Table II). This response can be attributed to enhanced NK cell activity following Mn treatment. A variety of target cells were found to be effective targets for $MnCl_2$-enhanced NK cell activity (Smialowicz et al., 1984b), including the relatively NK-insensitive P815 mastocytoma target (Wiltrout et al., 1982). A role for macrophage cytotoxicity in these experiments with spleen cells is highly unlikely because of the brief (4 h) incubation period employed and because of the absence of similar spontaneous activity against the non-NK target CRBC.

While $MnCl_2$ enhances NK and macrophage activity following in vivo exposure and NK activity following in vitro exposure (manuscript in preparation), a single injection of Mn failed to affect the lymphoproliferative response of spleen cells to T or B cell mitogens (data not shown). Furthermore, at the Mn levels employed in the present and earlier studies (Rogers et al., 1983; Smialowicz et al., 1984b), no effects were observed in the primary antibody response to sheep erythrocytes or in the in vitro generation of allogeneic cytotoxic T lymphocytes (data not shown). These results are not totally unexpected. Although IFN is known to affect the primary response to antigens, the generation of cytotoxic T lymphocytes and the mitogen-stimulated lymphoproliferation of spleen cells (De Mayer and De Mayer-Guignard, 1982), it does so only at much higher concentrations than those which were produced following Mn treatment.

The present results suggest that $MnCl_2$ enhances macrophage function through the induction of interferon, just as Mn augments NK cell activity through interferon (Rogers et al., 1983; Smialowicz et al., 1984b). The mechanism(s) by which Mn induces the production of interferon remains to be determined. It is possible that nonspecific inflammation, which occurs following an intramuscular injection of $MnCl_2$, may play a role in interferon induction. However, our earlier work with NK cell activity suggests that the observed effects appear to be Mn-specific (Rogers et al., 1983).

Manganese is an essential element and is present in all living organisms. It serves as a cofactor in a number of enzymatic reactions, particularly those that are involved in phosphorylation reactions and in cholesterol and fatty acid synthesis (Williams, 1967). Because it is so ubiquitous in the food chain, manganese deficiency has not been recognized in man (Beliles, 1979). Nevertheless, experimental manganese deficiencies in animals have been reported to result in skeletal abnormalities, defective ovulation, testicular degeneration, infant mortality, neonatal ataxia, and decreased glucose utilization (Shamberger, 1979). In the light of the work presented here, it would be interesting to determine if manganese deficiency in any way effects the immune system, as does a zinc deficiency (Beach et al., 1982; Fernandes et al., 1979).

Manganese has also been shown to be an effective inhibitor of metallic and nonmetallic carcinogenesis (Sunderman et al., 1976; Sunderman, 1979) and cell transformation (Costa et al., 1981). It also acts to increase the potency of a lymphosarcoma vaccine (McCollester, 1982), protects mice against endotoxin-induced mortality (Snyder et al., 1977) and inhibits LPS toxicity for macrophages (Patierno et al., 1983). The results presented here, as well as the intriguing results reported by others, suggest that manganese may play a significant role as an immunomodulator.

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